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<p>(21) International Application Number: PCT/DK93/00225 (22) International Filing Date: 5 July 1993 (05.07.93) (30) Priority data: 0888/92 6 July 1992 (06.07.92) DK (71) Applicant (for all designated States except US): NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bags- vaerd (DK). (72) Inventors; and (75) Inventors/Applicants (for US only) : SVENDSEN, Allan [DK/DK]; Bakkeleddet 28, DK-3460 Birkerød (DK). PATHAR, Shamkant, Anant [DK/DK]; Christoffers Allé 91, DK-2800 Lyngby (DK). EGEL-MITANI, Michi [DK/DK]; Goengesletten 31, DK-2950 Vedbæk (DK). BORCH, Kim [DK/DK]; Klerkegade 12.2.tv., DK-1808 Copenhagen K (DK). CLAUSEN, Ib, Groth [DK/DK]; Fyrrestien 6, DK-3400 Hillerød (DK). HANSEN, Mog- ens, Trier [DK/DK]; Mosevang 9, DK-3450 Lyngø (DK).</p>	<p>(81) Designated States: BR, JP, KR, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>	
<p>(54) Title: C. ANTARCTICA LIPASE AND LIPASE VARIANTS</p>		
<p>(57) Abstract</p> <p>A lipase variant of a parent lipase comprising a trypsin-like catalytic triad including an active serine located in a predominantly hydrophobic, elongated binding pocket of the lipase molecule and, located in a critical position of a lipid contact zone of the lipase structure, an amino acid residue different from an aromatic amino acid residue, which amino acid residue interacts with a lipid substrate at or during hydrolysis, in which lipase variant said amino acid residue has been replaced by an aromatic amino acid residue so as to confer to the variant an increased specific activity as compared to that of the parent lipase. The parent lipase may be a <i>C. antarctica</i> lipase A essentially free from other substances from <i>C. antarctica</i>, which comprises the amino acid sequence shown in SEQ ID No. 2, or a variant of said lipase which (1) has lipase activity, (2) reacts with an antibody reactive with at least one epitope of <i>C. antarctica</i> lipase A having the amino acid sequence SEQ ID No. 2, and/or (3) is encoded by a nucleotide sequence which hybridizes with an oligonucleotide probe prepared on the basis of the full or partial nucleotide sequence shown in SEQ ID No. 1 encoding the <i>C. antarctica</i> lipase A.</p>		

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C. antarctica lipase and lipase variants.

FIELD OF THE INVENTION

The present invention relates to novel lipase enzyme variants with improved properties, DNA constructs coding for the expression of said variants, host cells capable of expressing the variants from the DNA constructs, as well as a method of producing the variants by cultivation of said host cells. Furthermore, the present invention relates to a recombinant essentially pure Candida antarctica lipase and variants thereof as well as a DNA sequence encoding the said lipase or variants thereof.

BACKGROUND OF THE INVENTION

A wide variety of lipases of microbial and mammalian origin are known. The amino acid sequence of many of these lipases have been elucidated and analyzed with respect to structural and functional elements important for their catalytic function, see, for instance, Winkler et al., 1990 and Schrag et al., 1991. It has been found that the lipase enzyme upon binding of a lipid substrate and activation undergoes a conformational change, which inter alia, results in an exposure of the active site to the substrate. This conformational change together with the presumed interaction between enzyme and substrate have been discussed by, inter alia, Brady et al., 1990, Brzozowski et al., 1991, Derewenda et al., 1992.

Based on the knowledge of the structure of a number of lipases, it has been possible to construct lipase variants having improved properties by use of recombinant DNA techniques. Thus, WO 92/05249 discloses the construction of certain lipase variants, in which the lipid contact zone has been modified so as to provide the variants with different substrate specificities and/or an improved accessibility of the active site of the

, lipase to a lipid substrate. The modifications involve changing the electrostatic charge, hydrophobicity or the surface conformation of the lipid contact zone by way of amino acid substitutions.

5 Although the structural and functional relationship of lipases have been the subject of a number of studies as described in the above cited references, the research has mainly focused on the macroscopic characteristics of the lipases upon substrate binding and activation, whereas the identity of the amino acids
10 actually involved in the substrate binding and catalytic activity has been discussed only to a lesser extent.

SUMMARY OF THE INVENTION

By sequence alignment analysis combined with analysis of the structure and activity of a number of lipases, the present
15 inventors have now surprisingly found that the presence of certain amino acids, especially tryptophan, in a critical position of the lipase seems to be important for optimal catalytic activity.

It is consequently an object of the present invention to modify
20 lipases which do not comprise such an amino acid residue in the critical position (which lipases in the present context are termed parent lipases) by replacing the amino acid residue located in this position with an amino acid residue which gives rise to a variant having an increased specific activity.

25 More specifically, in one aspect the present invention relates to a lipase variant of a parent lipase comprising a trypsin-like catalytic triad including an active serine located in a predominantly hydrophobic, elongated binding pocket of the lipase molecule and, located in a critical position of a lipid
30 contact zone of the lipase structure, an amino acid residue different from an aromatic amino acid residue, which interacts

,with a lipid substrate at or during hydrolysis, in which lipase variant said amino acid residue has been replaced by an aromatic amino acid residue so as to confer to the variant an increased specific activity as compared to that of the parent s lipase.

In the present context, the term "trypsin-like" is intended to indicate that the parent lipase comprises a catalytic triad at the active site corresponding to that of trypsin, i.e. the amino acids Ser, His and one of Asp, Glu, Asn or Gln.

10 Lipases degrades triglycerides down to fatty acids, glycerol and di- and/or monoglycerides. The lipase action is depending on interfacial activation of the lipase in the presence of substrate surfaces. On activation lipases change their conformation in such a manner that their surface hydrophobicity in an 15 area around the active site is increased. The interfacial activation of lipases is e.g. discussed by Tilbeurgh et al. (1993).

All lipases studied until now have been found to comprise at least one surface loop structure (also termed a lid or a flap) 20 which covers the active serine when the lipase is in inactive form (an example of such a lipase is described by Brady et al., 1990). When the lipase is activated, the loop structure is shifted to expose the active site residues, creating a surface surrounding the active site Ser, which has an increased surface 25 hydrophobicity and which interacts with the lipid substrate at or during hydrolysis. For the present purpose, this surface is termed the "lipid contact zone", intended to include amino acid residues located within or forming part of this surface, optionally in the form of loop structures. These residues may 30 participate in lipase interaction with the substrate at or during hydrolysis where the lipase hydrolyses triglycerides from the lipid phase when activated by contact with the lipid surface.

The lipid contact zone contains a binding area (a so-called binding pocket) for the lipid substrate which is the part of the lipid contact zone to which the lipid substrate binds before hydrolysis. This binding area again contains a so-called hydrolysis pocket, which is situated around the active site Ser, and in which the hydrolysis of the lipid substrate is believed to take place. In all known lipases to day the lipid contact zone is easily recognized, e.g. from a three-dimensional structure of the lipase created by suitable computer programs. The conformation of an inactive and activated lipase, respectively, is shown in Fig. 1 which is further discussed below.

In the present context, the "critical position" of the lipase molecule is the position in the lipid contact zone of the lipase molecule, which is occupied by an amino acid residue which interacts with the lipid substrate and which is different from an aromatic amino acid residue.

In another aspect the present invention relates an C. antarctica lipase A which is essentially free from other C. antarctica substances and which comprises the amino acid sequence identified in SEQ ID No. 2 or a variant thereof which

- 1) has lipase activity,
- 2) reacts with an antibody reactive with at least one epitope of the C. antarctica lipase having the amino acid sequence shown in SEQ ID No. 2, and/or
- 3) is encoded by a nucleotide sequence which hybridizes with an oligonucleotide probe prepared on the basis of the full or partial nucleotide sequence shown in SEQ ID No. 1 encoding the C. antarctica lipase A.

The C. antarctica lipase A of the invention has a number of desirable properties including a high thermostability and

activity at acidic pH and may advantageously be produced by use of recombinant DNA techniques, e.g. using the procedures described below. Thus, the lipase A of the invention may be obtained in a higher purity and a higher amount than the C. antarctica lipase A purified from wild type C. antarctica which is described in WO 88/02775.

Furthermore, the present invention relates to a DNA sequence encoding the C. antarctica lipase A having the amino acid sequence identified in SEQ ID No. 2 or a modification of said DNA sequence encoding a variant of the C. antarctica lipase A as defined above.

In the present context "C. antarctica lipase A" is used interchangeably with "lipase A" and the variant of the C. antarctica lipase A is termed "lipase A variant".

The present invention also relates to a DNA construct comprising a DNA sequence encoding a lipase variant as indicated above or a DNA sequence encoding the C. antarctica lipase A, a recombinant expression vector carrying said DNA construct, a cell transformed with the DNA construct or the expression vector, as well as a method of producing a lipase variant of the invention by culturing said cell under conditions conducive to the production of the lipase variant, after which the lipase variant is recovered from the culture.

It will be understood that lipase variants of the present invention having an increased specific activity as compared to their parent lipases may be used for the same purposes as their parent lipases, advantageously in a lower amount due to their higher specific activity.

Accordingly, the present invention relates to the use of a lipase variant of the invention as a detergent enzyme; as a digestive enzyme; in ester hydrolysis, ester synthesis or interesterification; or the use of the lipase variant to avoid pitch

trouble arising, e.g., in processes for preparing mechanical pulp and in paper-making processes using mechanical pulp.

DETAILED DISCLOSURE OF THE INVENTION

As indicated above, the present inventors have found that the presence of certain aromatic amino acids, especially tryptophan, located in the lipid contact zone of the lipase molecule is important for optimal catalytic activity.

The importance of the presence of an aromatic amino acid residue and in particular a tryptophan residue was found in connection with a study of mutants of a Humicola lanuginosa lipase which comprises a tryptophan residue at the critical position in the lipid contact zone, i.e. the amino acid number 89 in the amino acid sequence of the H. lanuginosa lipase published in EP 0 305 216. In the H. lanuginosa mutants this tryptophan residue was replaced by phenylalanine, tyrosine, histidine, isoleucine, glutamic acid and glycine, respectively. It was found that the specific activity of these mutants decreased (in the order indicated above) from 100% of the wild type lipase to about 10% of the phenylalanine mutant and down to about 2% for the glycine mutant.

Without being limited to any theory it is presently believed that the amino acid residue present in the critical position, e.g. on top of or in the proximity of the active serine, may be involved in a) stabilization of the tetrahedral intermediate formed from the lipase and the substrate during the activation of the lipase, and b) in the activation of the replacement of the lid region covering the active serine in the inactive enzyme. When tryptophan is present in this position, it is contemplated that optimal performance with respect to a) as well as b) above is obtained. Thus, it is believed that tryptophan gives rise to the formation of the most stable tetrahedral intermediate (which means a lowering of the activation energy

,needed for the catalysis to take place), and further improves the performance of the enzyme with respect to the activation of the lid opening which is essential for any catalysis to take place.

5 In connection with a) above it has been observed that the best acting lipase variants contain an unsaturated ring system in the side-chain. The far the biggest unsaturated system is tryptophan, then tyrosine, phenylalanine and histidine. These sidechains have a pi-electron system ("the unsaturation") that
10 could be important for the proton transfer in the catalysis resulting in a lower activation energy for creating the tetrahedral intermediate where proton transfer has taken place from active site histidine to serine to the oxyanion hole created after lid activation and opening.

15 From the above theoretical explanation it will be understood that the optimal amino acid to be present in the critical position, e.g. on top of or in the proximity of the active serine, is tryptophan. However, when the parent lipase is one which does not contain any aromatic amino acid residue or any
20 amino acid residue with an unsaturated ring system in the side-chain in this position, such amino acids may advantageously be substituted into this position.

Thus, when the parent lipase, in the critical position, has an amino acid residue which does not comprise an unsaturated ring
25 system in the side-chain, an amino acid residue having such an unsaturated ring-system, e.g. an aromatic amino acid (tryptophan, tyrosine, phenylalanine or histidine) may be substituted into the critical position. When the amino acid residue in the critical position of the parent lipase is
30 histidine, it may advantageously be replaced by phenylalanine, tyrosine and most preferably tryptophan, when the amino acid residue is tyrosine, it may advantageously be replaced by phenylalanine and most preferably tryptophan, and when the

amino acid residue is phenylalanine it may advantageously be replaced by tryptophan.

While the critical position in some lipases is contemplated to be any position within the lipid contact zone, the critical position will normally be located in the binding pocket of the lipase molecule, and preferably in the hydrolysis pocket thereof. For most lipases it is believed that the critical amino acid residue is positioned on top of or in the proximity of the active site.

10 The amino acid residue occupying this position may be identified in any lipase by 1) sequence alignment studies in which the amino acid sequence of the lipase in question is aligned with the amino acid sequence of other lipases, in which the amino acid residue positioned on top of or in the proximity of
15 the active serine has been identified, so as to identify the presumed position of said amino acid residue, and/or 2) an analysis of the three-dimensional structure of the lipase in question using standard display programmes such as INSIGHT (Biosym Technologies Inc., San Diego, USA), so as identify the
20 amino acid sequence on top of or in the proximity of the active serine.

More specifically, on the basis of a computer program such as INSIGHT displaying lipase coordinates in accordance with well-known technology, it is simple to point out which part of the
25 lipase which contains the lipid contact zone. 1/ if the structure of the lipase is in a non-activated form, the lipid contact zone is identified by the direction of sidechains of the active site Ser. 2/ if the structure is in the activated form one may additionally base the identification on a colour-
30 ing of all hydrophobic residues in a colour different from the other residues. By this procedure in which a cpk model of the structure is created, the hydrophobic surface specific for the lipid contact zone may be identified. The active site Ser is located within this more hydrophobic part of the molecule.

In some lipases the critical amino acid residue is located in the surface loop structure covering the active site, or in one or more of the surface loop structures found to form part of the surface of the lipid contact zone, such as of the binding pocket or hydrolysis pocket.

Although the critical position is normally considered to be constituted of only one amino acid residue it may be advantageous to replace two or more residues, preferably with a tryptophan residue as explained above, in order to obtain a further increased specific activity.

It is contemplated that it is possible to increase the specific activity of parent lipases which do not have a tryptophan residue in the critical position at least 2 times, such as at least 3 and preferably at least 4 or even 5, 6 or 7 times by modifications as disclosed herein.

It is contemplated that lipase variants as defined herein having an increased substrate specificity may be prepared on the basis of parent lipases of various origins. Thus, the parent lipase may be a microbial lipase or a mammalian lipase.

When the parent lipase is a microbial lipase, it may be selected from yeast, e.g. Candida, lipases, bacterial, e.g. Pseudomonas, lipases or fungal, e.g. Humicola or Rhizomucor lipases.

One preferred lipase variant is one, in which the parent lipase is derived from a strain of Candida antarctica, in particular one in which the parent lipase is lipase A of C. antarctica, preferably the one which has the amino acid sequence shown in SEQ ID No. 2 or a lipase A variant thereof as defined herein. The lipase variant of this C. antarctica lipase A preferably has the amino acid sequence shown in SEQ ID No. 2 in which the phenylalanine 139 of the parent lipase has been replaced by a tryptophan residue. The construction of this variant and the

analysis of the properties thereof is discussed in Example 3, 5 and 6.

A lipase variant of the invention may, as mentioned above, be prepared on the basis of a parent lipase derived from a strain of a Pseudomonas species, e.g. Ps. fragi. An example of a suitable Ps. fragi lipase which has an amino acid residue different from tryptophan positioned on top of or in the proximity of the active serine, is the one described by Aoyama et al., 1988. A lipase variant according to the present invention may be constructed by replacing the phenylalanine residue 29 in the amino acid sequence of said lipase shown in SEQ ID No. 3 by a tryptophan residue.

An example of a fungal lipase suitable as a parent lipase for the construction of a lipase variant of the invention is one derived from Rhizopus, especially from R. delemar or R. niveus, the amino acid sequence of which latter is disclosed in, e.g., JP 64-80290. In order to construct a lipase variant according to the present invention from this parent lipase, the alanine residue at position 117 is to be replaced with an aromatic amino acid residue such as tryptophan. The sequence alignment of the R. niveus lipase sequence (SEQ ID No. 5) and an Rhizomucor miehei lipase sequence (containing a tryptophan residue) (SEQ ID No. 4) is illustrated below. From this alignment the critical position of the R. niveus lipase may be determined.

25	SEQUENCE	10	20	30	40	50	60	Res#
		----- ----- ----- ----- ----- -----						
	<u>mucor</u>	-----SIDGGIRAATSQEI NELTYITLSANSYCRIV						32
	<u>niveus</u>	DONLVGGMILDLPDAPFISLSSSINSASDGGKVAATTAQIQEFIKYAGIAATAVCRSV						60
30	SEQUENCE	70	80	90	100	110	120	Res#
		----- ----- ----- ----- ----- -----						
	<u>mucor</u>	IPGATWDCIHCDAE-DLKI IKTWSTLTYDINAMVARGDSEKTTYIVFRGSSSIRNIAD						91
	<u>niveus</u>	VPGNKWDCVQOKWVPDGIITTFISLSDINGVLRSDKQKTTYLVFRGINSFRSAITD						120

SEQUENCE	130	140	150	160	170	180	Res#		
	----- ----- ----- ----- ----- -----								
mucor	LTFV	VSYP	PVSG	IKVH	KGF	LD	SYGEVQNELVATVLDQFKQYPSYKVAVTGHSILGGATAL 151		
niveus	IVFN	FSYK	PVKG	AKVH	AGFL	SSYEQV	VNDYFPVWQEQLIHAPTYKVVIVTGHSLGGAQAL 180		
5 SEQUENCE	190	200	210	220	230	240	Res#		
	----- ----- ----- ----- ----- -----								
mucor	LCAL	GLYQ	REEG	LSSN	LF	LYTQ	QPRVGDPAFANYVWSIGIPYRRTVNERDIVPHLPPA 211		
niveus	LACM	DLYQ	REPRL	SPK	NLSI	FIVG	PRVGNPTIFAYYVESTIGIPFQKIVHKRDIVPHVPPQ 240		
SEQUENCE	250	260	270	280	290	300	Res#		
	----- ----- ----- ----- ----- -----								
10									
mucor	AFGL	HAGE	EYWT	DNSP	ETVQ	VCIS	DLEISDCS	NSIVPFTS	VLDHLSYFGINIGLCS 269
niveus	SFGF	LHPG	VESW	IKSG	ISN	VQIC	ISEIEIKDCS	NSIVPFTS	ILDHLSYFDINEGSCL 297

The present inventors have surprisingly found that non-pancreatic lipases such as gastric, lingual, or hepatic lipases have the common feature that the amino acid residue which has been identified to be the one located in the critical position of the lipase molecule, normally on top of or in the proximity of the active serine, is different from tryptophan. This is in contrast to pancreatic lipases which generally have been found to have a tryptophan residue in this position. Thus, in the present context, non-pancreatic mammalian lipases may advantageously be used as "parent lipases" for the construction of lipase variants of the invention.

Accordingly, lipase variants as disclosed herein which is of mammalian origin is advantageously prepared from a parent lipase of non-pancreatic, such as gastric, lingual or hepatic origin. Such mammalian lipases may be derived from humans, rats, mice, pigs, dogs or other mammals. Specific examples of such mammalian lipases includes a rat lingual lipase having the sequence identified as A23045 (Docherty et al., 1985), a rat hepatic lipase having the sequence identified as A27442

(Komaromy and Schotz, 1987), a human hepatic lipase having the sequence identified as A33553 (Datta et al., 1988), a human gastric lipase having the sequence identified as S07145 (Bodmer et al., 1987), and a Bio Salt Activated Lipase (BSAL) having the sequence identified as A37916 (Baba et al., 1991) all of which were analysed with respect to the critical position in the sequence alignment analysis illustrated below. The pancreatic lipases included in this sequence alignment study were a murine pancreatic lipase, A34671 (Grusby et al., 1990), a porcine pancreatic lipase, A00732 (Caro et al., 1981), a human pancreatic lipase, A34494 (Lowe et al., 1989), and a canine pancreatic lipase having the sequence B24392 (Mickel et al., 1989). The amino acid sequences of each of the lipases mentioned have the accession numbers listed above and are available from publically available databases.

	42	89
	A37916 TYGDEDCLYL NIWVQGRK. ..QVSRDLPV MIWIYGGAFI MGSGHGANFL	
	A23045 EVVTEDGYIL GVYRIPHGKN NSENIGKRPV VYLOHGLIAS AT..NWIANL	
	S07145 EVVTEDGYIL EVNRIPYGKK NSGNIGQRPV VFLOHGLLAS AT..NWLSNL	
20	B24392 TNKNPNNFQT LLPSPSTIE ASNFQIDKKT RFTIHGFINK GE.ENWLLDM	
	A34494 TNENPNNFQE VA.ADSSSIS GSNFKTNRKT RFLIHGFIDK GE.ENWLANV	
	A34671 TNENPNNYQI ISATDPATIN ASNFQIDRKT RFLIHGFIDK GE.EGWLLDM	
	A00732 TNQNNNYQE LV.ADPSTTT NSNFRMDRKT RFLIHGFIDK GE.EDWLSNI	
	A33553 GEINQ..GOQ IRINHPDILQ EOGFNSSLPL VMLIHGWSVD GVLENWIWQM	
25	A27442 KDESDRLGQQ IRLPHPEILQ EOGFNSSHPL VMLIHGWSVD GLEIWIWKI	
	90	130
	A37916 NNYLYDGEEI ATRGNVIVVT FNYRVGPLGF LSTGDANLPG NYGLRDOHMA	
	A23045 PNNSLAFMLA DAGYDVLGN SRGNIWSRKN VYSPDSVEF WAFSFDMAK	
	S07145 PNNSLAFILA DAGYDVLGN SRGNIWARRN LYSPDSVEF WAFSFDMAK	
30	B24392 CKRMFKVEE.VN CICVDWKKGS QISYTOAANN VRVVGQVVAQ	
	A34494 CKNLFKVES.VN CICVDWKGGS RIGYTOASQN IRIVGAEVAY	
	A34671 CKRMFQVEK.VN CICVDWKRGS RIEYTOASYN TRVVGAEIAP	
	A00732 CKNLFKVES.VN CICVDWKGGS RIGYTOASQN IRIVGAEVAY	
	A33553 VAALKSQPAQ P.....VN VGLVDWITLA HDHYTIAVRN TRLVGKEVAA	
35	A27442 VGALKSRQSQ P.....VN VGLVDWISLA YQHYAIAVRN TRVVGQEVAA	

	131			175
	A37916	IAWVKRNI.A	AFGGDPNNIT	LFGESAGGAS VSLQTLSPYN K...GLIRRA
	A23045	YDLPATINFI	VQKTGQEKIH	YVGHSSQGITI GFIAPSTNPT L..AKKIKTF
	S07145	YDLPATIDFI	VKKTGQKQLH	YVGHSSQGITI GFIAPSTNPS L..AKRIKTF
5	B24392	MLSMLS...A	NYSYSPSQVQ	LIGHSLGAHV AGEAGSRTPG ...LGRITGL
	A34494	FVEFLQ...S	AFGYSPSNVH	VIGHSLGAHA AGEAGRRING T..IGRITGL
	A34671	LVQVLS...T	EMGYSPENVH	LIGHSLGSHV AGEAGRRELE H..VGRITGL
	A00732	FVEVLK...S	SLGYSPSNVH	VIGHSLGSHA AGEAGRRING T..IERITGL
	A33553	ILRWLE...E	SVQLSRSHVH	LIGYSLGAHV SGFAGSSIGG THKIGRITGL
10	A27442	LLLWLE...E	SMKFSRSKVH	LIGYSLGAHV SGFAGSSMGG KKKIGRITGL

	176			220
	A37916	ISQSGVALSP	WVIQKN....	..PLFWAKKV AEKVGCPVGD AARMAQCLKV
15	A23045	YALAPVATVK	YTQSPLKKIS	FIPTFLFKLM FGKRMFLPHT YFDDFLGTEV
	S07145	YALAPVATVK	YTKSLINKLR	FVQSLFKFI FGDKIFYPHN FFDQFLATEV
	B24392	DPVEASFQGT	PEEVRLD...	..PTDADFVD VIHDAAPLI PFLGFGTSQQ
	A34494	DPAEPCFQGT	PELVRLD...	..PSDAKFVD VIHIDGAPIV PNLGFGMSQV
	A34671	DPAEPCFQGL	PEEVRLD...	..PSDAMFVD VIHIDSAPII PYLGFMSQK
20	A00732	DPAEPCFQGT	PELVRLD...	..PSDAKFVD VIHDAAPII PNLGFGMSQT
	A33553	DAAGPLFEQS	APSNRLS...	..PDDASFVD AIHTFIREHM GLSVGK.QP
	A27442	DPAGPMFEGT	SPNERLS...	..PDDANFVD AIHTFIREHM GLSVGK.QP

Z = Flap region

25		221		270
	A37916	TDFRALITLAY	KVPLAGLEYD	MLHYVGFVPV IDGDFIPADP INLYANAADI
	A23045	CSREVDLLC	SNILFIFCGF	DKKNLNVSRF DVYLGHNPAG TSVQDFLHWA
	S07145	CSREMLNLLC	SNALFIIQGF	DSKNFNISRL DVYLSHNPAG TSVQNMFWHT
	B24392	MGHLDFFPNG	GEEMPGCKKN	ALSQIVNLDG IWEGTRDFVA CNHLRSYKYY
30	A34494	VGHLDFFPNG	GVEMPGCKKN	ILSQIVDIDG IWEGTRDFAA CNHLRSYKYY
	A34671	VGHLDFFPNG	GKEIPGQCKN	ILSTIVDING IWEGTRNFAA CNHLRSYKYY
	A00732	VGHLDFFPNG	GKQMPGQCKN	ILSQIVDIDG IWEGTRDFVA CNHLRSYKYY
	A33553	IGHYDFYPNG	GSPQPGCHF	ELYRHIAQHG FNATTQTIK. CSHERSVHLF
	A27442	IAHYDFYPNG	GSPQPGCHF	ELYKHIAEHG LNATTQTIK. CAHERSVHLF

As mentioned above the present invention also relates to a C. antarctica lipase A essentially free from other C. antarctica substances, which has the amino acid sequence shown in SEQ ID No. 2 or a variant thereof which

- 5 1) has lipase activity,
- 2) reacts with an antibody reactive with at least one epitope of C. antarctica lipase A having the amino acid sequence shown in SEQ ID No. 2, and/or
- 3) is encoded by a nucleotide sequence which hybridizes with an
10 oligonucleotide probe prepared on the basis of the full or partial nucleotide sequence shown in SEQ ID No. 1 encoding the C. antarctica lipase A.

In the present context, the term "variant" is intended to indicate a lipase A variant which is derived from the C.
15 antarctica lipase A having the amino acid sequence shown in SEQ ID No. 2, or a naturally occurring variant. Typically, the variant differ from the native lipase A by one or more amino acid residues, which may have been added or deleted from either or both of the N-terminal or C-terminal end of the lipase,
20 inserted or deleted at one or more sites within the amino acid sequence of the lipase or substituted with one or more amino acid residues within, or at either or both ends of the amino acid sequence of the lipase.

Furthermore, the variant of the invention has one or more of
25 the characterizing properties 1)-3) mentioned above. Property 1), i.e. the "lipase activity" of the variant may be determined using any known lipase assay, e.g. the Standard LU assay described in the Methods section below.

Property 2), i.e. the reactivity of the variant of the inven-
30 tion with an antibody raised against or reactive with at least one epitope of the C. antarctica lipase A having the amino acid

sequence shown in SEQ ID No. 2 below may be determined by polyclonal antibodies produced in a known manner, for instance by immunization of a rabbit with the C. antarctica lipase A of the invention. The antibody reactivity may be determined using assays known in the art, examples of which are Western Blotting or radial immunodiffusion assay.

Property 3) above, involving hybridization, may be performed using an oligonucleotide probe prepared on the basis of the full or partial cDNA sequence encoding the C. antarctica lipase A, the amino acid sequence of which is identified in SEQ ID No. 2, as a hybridization probe in a hybridization experiment carried out under standard hybridization conditions. For instance, such conditions are hybridization under specified conditions, e.g. involving presoaking in 5xSSC and prehybridizing for 1h at -40°C in a solution of 20% formamide, 5xDenhardt's solution, 50mM sodium phosphate, pH 6.8, and 50µg of denatured sonicated calf thymus DNA, followed by hybridization in the same solution supplemented with 100µM ATP for 18h at -40°C, or other methods described by e.g. Sambrook et al., 1989.

The nucleotide sequence on the basis of which the oligonucleotide probe is prepared is conveniently the DNA sequence shown in SEQ ID No. 1.

As stated above in a further aspect the present invention relates to a DNA sequence encoding C. antarctica lipase A having the amino acid sequence shown in SEQ ID No. 2 or a modification of said DNA sequence which encodes a variant of C. antarctica lipase A which

- 1) has lipase activity,
- 2) reacts with an antibody reactive with at least one epitope of the C. antarctica lipase A having the amino acid sequence shown in SEQ ID No. 2, and/or

, 3) is encoded by a nucleotide sequence which hybridizes with an oligonucleotide probe prepared on the basis of the full or partial nucleotide sequence shown in SEQ ID No. 1 encoding the C. antarctica lipase A.

5 Examples of suitable modifications of the DNA sequence are nucleotide substitutions which do not give rise to another amino acid sequence of the encoded enzyme, but which may correspond to the codon usage of the host organism into which the DNA sequence is introduced or nucleotide substitutions
10 which do give rise to a different amino acid sequence, without, however, impairing the above stated properties of the enzyme. Other examples of possible modifications are insertion of one or more nucleotides into the sequence, addition of one or more nucleotides at either end of the sequence and deletion of one
15 or more nucleotides at either end of or within the sequence.

Methods of preparing lipase variants of the invention

Several methods for introducing mutations into genes are known in the art. After a brief discussion of cloning lipase-encoding DNA sequences, methods for generating mutations at specific
20 sites within the lipase-encoding sequence will be discussed.

Cloning a DNA sequence encoding a lipase

The DNA sequence encoding a parent lipase or the C. antarctica lipase A as defined herein may be isolated from any cell or microorganism producing the lipase in question by various
25 methods, well known in the art. First a genomic DNA and/or cDNA library should be constructed using chromosomal DNA or messenger RNA from the organism that produces the lipase to be studied. Then, if the amino acid sequence of the lipase is known, homologous, labelled oligonucleotide probes may be
30 synthesized and used to identify lipase-encoding clones from a genomic library of bacterial DNA, or from a fungal cDNA library. Alternatively, a labelled oligonucleotide probe containing sequences homologous to lipase from another strain of bacteria or fungus could be used as a probe to identify

lipase-encoding clones, using hybridization and washing conditions of lower stringency.

Yet another method for identifying lipase-producing clones would involve inserting fragments of genomic DNA into an expression vector, such as a plasmid, transforming lipase-negative bacteria with the resulting genomic DNA library, and then plating the transformed bacteria onto agar containing a substrate for lipase. Those bacteria containing lipase-bearing plasmid will produce colonies surrounded by a halo of clear agar, due to digestion of the substrate by secreted lipase.

Alternatively, the DNA sequence encoding the enzyme may be prepared synthetically by established standard methods, e.g. the phosphamidite method described by S.L. Beaucage and M.H. Caruthers (1981) or the method described by Matthes et al. (1984). According to the phosphamidite method, oligonucleotides are synthesized, e.g. in an automatic DNA synthesizer, purified, annealed, ligated and cloned in appropriate vectors.

Finally, the DNA sequence may be of mixed genomic and synthetic, mixed synthetic and cDNA or mixed genomic and cDNA origin prepared by ligating fragments of synthetic, genomic or cDNA origin (as appropriate), the fragments corresponding to various parts of the entire DNA sequence, in accordance with standard techniques. The DNA sequence may also be prepared by polymerase chain reaction (PCR) using specific primers, for instance as described in US 4,683,202 or R.K. Saiki et al. (1988).

Site-directed mutagenesis of the lipase-encoding sequence

Once a lipase-encoding DNA sequence has been isolated, and desirable sites for mutation identified, mutations may be introduced using synthetic oligonucleotides. These oligonucleotides contain nucleotide sequences flanking the desired mutation sites; mutant nucleotides are inserted during oligonucleotide synthesis. In a specific method, a single-stranded gap of DNA, bridging the lipase-encoding sequence, is created in a vector

carrying the lipase gene. Then the synthetic nucleotide, bearing the desired mutation, is annealed to a homologous portion of the single-stranded DNA. The remaining gap is then filled in with DNA polymerase I (Klenow fragment) and the construct is ligated using T4 ligase. A specific example of this method is described in Morinaga et al. (1984). U.S. Patent number 4,760,025 discloses the introduction of oligonucleotides encoding multiple mutations by performing minor alterations of the cassette, however, an even greater variety of mutations can be introduced at any one time by the Morinaga method, because a multitude of oligonucleotides, of various lengths, can be introduced.

Another method of introducing mutations into lipase-encoding sequences is described in Nelson and Long (1989). It involves the 3-step generation of a PCR fragment containing the desired mutation introduced by using a chemically synthesized DNA strand as one of the primers in the PCR reactions. From the PCR-generated fragment, a DNA fragment carrying the mutation may be isolated by cleavage with restriction endonucleases and reinserted into an expression plasmid.

Expression of lipase variants

According to the invention, a C. antarctica lipase A-coding sequence or a mutated lipase-coding sequence produced by methods described above or any alternative methods known in the art, can be expressed, in enzyme form, using an expression vector which typically includes control sequences encoding a promoter, operator, ribosome binding site, translation initiation signal, and, optionally, a repressor gene or various activator genes. To permit the secretion of the expressed protein, nucleotides encoding a "signal sequence" may be inserted prior to the lipase-coding sequence. For expression under the direction of control sequences, a target gene to be treated according to the invention is operably linked to the control sequences in the proper reading frame. Promoter sequences that can be incorporated into plasmid vectors, and

, which can support the transcription of the mutant lipase gene, include but are not limited to the prokaryotic β -lactamase promoter (Villa-Kamaroff et al. (1978) and the tac promoter (DeBoer, et al., 1983). Further references can also be found 5 in "Useful proteins from recombinant bacteria" (1980).

According to one embodiment a strain of Bacillus, e.g. B. subtilis, B. licheniformis or B. lentus, or a strain of E. coli is transformed by an expression vector carrying the lipase A or the mutated DNA. If expression is to take place in a secreting 10 microorganism such as B. subtilis a signal sequence may follow the translation initiation signal and precede the DNA sequence of interest. The signal sequence acts to transport the expression product to the cell wall where it is cleaved from the product upon secretion. The term "control sequences" as 15 defined above is intended to include a signal sequence, when is present.

The lipase or lipase variants of the invention may further be produced by using a yeast cell has a host cell. Examples of suitable yeast host cells include a strain of Saccharomyces, 20 such as S. cerevisiae, or a strain of Hansenula, e.g. H. polymorpha or Pichia, e.g. P. pastoris.

In a currently preferred method of producing lipase A or lipase variants of the invention, a filamentous fungus is used as the host organism. The filamentous fungus host organism may 25 conveniently be one which has previously been used as a host for producing recombinant proteins, e.g. a strain of Aspergillus sp., such as A. niger, A. nidulans or A. oryzae. The use of A. oryzae in the production of recombinant proteins is extensively described in, e.g. EP 238 023.

30 For expression of lipase variants in Aspergillus, the DNA sequence coding for the lipase A or the lipase variant is preceded by a promoter. The promoter may be any DNA sequence exhibiting a strong transcriptional activity in Aspergillus and

may be derived from a gene encoding an extracellular or intracellular protein such as an amylase, a glucoamylase, a protease, a lipase, a cellulase or a glycolytic enzyme.

Examples of suitable promoters are those derived from the gene encoding A. oryzae TAKA amylase, Rhizomucor miehei aspartic proteinase, A. niger neutral α -amylase, A. niger acid stable α -amylase, A. niger glucoamylase, Rhizomucor miehei lipase, A. oryzae alkaline protease or A. oryzae triose phosphate isomerase.

10 In particular when the host organism is A. oryzae, a preferred promoter for use in the process of the present invention is the A. oryzae TAKA amylase promoter as it exhibits a strong transcriptional activity in A. oryzae. The sequence of the TAKA amylase promoter appears from EP 238 023.

15 Termination and polyadenylation sequences may suitably be derived from the same sources as the promoter.

The techniques used to transform a fungal host cell may suitably be as described in EP 238 023.

To ensure secretion of the lipase A or the lipase variant from the host cell, the DNA sequence encoding the lipase variant may be preceded by a signal sequence which may be a naturally occurring signal sequence or a functional part thereof or a synthetic sequence providing secretion of the protein from the cell. In particular, the signal sequence may be derived from a gene encoding an Aspergillus sp. amylase or glucoamylase, a gene encoding a Rhizomucor miehei lipase or protease, or a gene encoding a Humicola cellulase, xylanase or lipase. The signal sequence is preferably derived from the gene encoding A. oryzae TAKA amylase, A. niger neutral α -amylase, A. niger acid-stable α -amylase or A. niger glucoamylase.

The medium used to culture the transformed host cells may be any conventional medium suitable for culturing Aspergillus cells. The transformants are usually stable and may be cultured in the absence of selection pressure. However, if the transformants are found to be unstable, a selection marker introduced into the cells may be used for selection.

The mature lipase protein secreted from the host cells may conveniently be recovered from the culture medium by well-known procedures including separating the cells from the medium by centrifugation or filtration, and precipitating proteinaceous components of the medium by means of a salt such as ammonium sulphate, followed by chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

It will be understood that the lipase variants of the invention are contemplated to be active towards the same type of substrates as their parent lipases, with an improved specific activity. Thus, the lipase variants of the invention are contemplated to be useful for the same purposes as their parent lipases.

Accordingly, lipase variants of the invention prepared from a parent lipase useful as a detergent enzyme may be used as an active ingredient in a detergent additive or a detergent composition.

Another contemplated use of lipase variants of the invention, is as digestive enzymes, e.g. in the treatment of cystic fibrosis.

A third use of the lipase variants of the invention, especially variants of C. antarctica lipases are in lipase-catalysed processes such as in ester hydrolysis, ester synthesis and interesterification. The use of lipases in these processes is discussed in detail in WO 88/02775 (Novo Nordisk A/S), the content of which is incorporated herein by reference. Further-

, more, as the C. antarctica is an unspecific lipase, it may be used for randomization, e.g. in the preparation of margarine. Also the lipase variants of the invention may be used to avoid pitch trouble that arises in the production process for mechanical pulp or in a paper-making process using mechanical pulp, e.g. as described in PCT/DK92/00025 (Novo Nordisk A/S), the content of which is incorporated herein by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention is described in the following with reference to the appended drawings, in which

Fig. 1 is a computer model showing the three-dimensional structure of the lipid contact zone of the H. lanuginosa lipase described in WO 92/05249 when the lipase is in inactive (closed) and active (open) form, respectively. "White" residues represent hydrophobic amino acids (Ala, Val, Leu, Ile, Pro, Phe, Trp, Gly and Met), "yellow" residues represent hydrophilic amino acids (Thr, Ser, Gln, Asn, Tyr and Cys), "blue" residues represent positively charged amino acids (Lys, Arg and His), and "red" residues represent negatively charged amino acids (Glu and Asp).

Figs. 2 and 3 illustrate the scheme for the construction of the expression plasmid pMT1229 (see Example 1).

The present invention is further illustrated in the following examples which are not intended, in any way, to limit the scope of the invention as claimed.

MATERIALS**Plasmids and microorganisms**

- pBoel777 (p777) (described in EP 0 489 718) .
p775 (the construction of which is described in EP 0 238 023)
5 pIC19H (Marsh et al., Gene 32 (1984), pp. 481-485)
pToC90 (described in WO 91/17243)
Aspergillus oryzae A1560: IFO 4177
E. coli MT172 (a K12 restriction deficient E. coli MC1000
derivative)

10 GENERAL METHODS**Site-directed in vitro mutagenesis of lipase genes**

The three different approaches described in WO 92/05249 may be used for introducing mutations into the lipase genes, i.e. the oligonucleotide site-directed mutagenesis which is described by
15 Zoller & Smith, DNA, Vol. 3, No. 6, 479-488 (1984), the PCR method as described in Nelson & Long, Analytical Biochemistry, 180, 147-151 (1989), and the so-called "cassette mutagenesis" technique, in which a segment between two restriction sites of the lipase-encoding region is replaced by a synthetic DNA frag-
20 ment carrying the desired mutation. Use of the latter technique is illustrated in Example 2.

Determination of lipase specific activity

Lipase activity was assayed using glycerine tributyrat as a substrate and gum-arabic as an emulsifier. 1 LU (Lipase Unit)
25 is the amount of enzyme which liberates 1 μ mol titratable butyric acid per minute at 30°C, pH 7.0. The lipase activity was assayed by pH-stat using Radiometer titrator VIT90, Radiometer, Copenhagen. Further details of the assay are given in Novo Analytical Method AF 95/5, available on request.

, EXAMPLES

EXAMPLE 1

Cloning of Candida antarctica lipase A

Chromosomal DNA of the C. antarctica strain LF058 (= DSM 3855
 5 deposited with the Deutsche Sammlung von Mikroorganismen (DSM)
 on September 29, 1986 under the terms of the Budapest Treaty,
 and further described in WO 88/02775) was prepared by opening
 of frozen cells by grinding with quartz and subsequent extrac-
 tion of DNA essentially as described by Yelton et al., (1984).
 10 The purified DNA was cut partially with Sau3A and, after
 agarose gel electrophoresis, fragments in the range of 3-9 kb
 were isolated. The sized Sau3A fragments were ligated into a
 BamHI-cut, dephosphorylated plasmid pBR322 (New England
 Biolabs). The ligation mix was transformed into the E. coli
 15 MT172. Approximately 50,000 transformant E. coli colonies were
 obtained, 80% of which contained an insert of LF058 DNA.

Using standard colony hybridization techniques (Maniatis et
 al., 1982) the colonies were screened with the ^{32}P -phosphory-
 lated oligonucleotide probe NOR 440 (SEQ ID No. 7). NOR 440 is
 20 a degenerated (64) 17 mer based on the N-terminal determined
 from mature C. antarctica lipase A (SEQ ID No. 2). 34 colonies
 appeared positive after wash at low stringency (41°C and 6 x
 SSC). Plasmids were prepared from these colonies and Southern
 analyzed after restriction with BstNI. The probe for the
 25 Southern was either the NOR 440 probe (SEQ ID No. 7) used for
 the colony hybridization (see above) or a ^{32}P -labelled probe NOR
 438 (SEQ ID No. 6). NOR 438 is an oligonucleotide (a guess mer)
 where, at 13 positions, a base has been chosen on the basis of
 codon use in yeasts and filamentous fungi.

30 AACCCATACGACGACCC
 T C T T T
 G
 T

NOR 440
 (SEQ ID No. 7)

GCTGCTCTGCCTAACCCCTTACGACGACCCTTTCTACACCACCCC NOR 438
T T T (SEQ ID No. 6)

guess positions indicated

5 Only one plasmid, PMT1076, contained a band which hybridised both to NOR 440 at low stringency (see above) and to NOR 438 at a somewhat higher stringency (55°C and 1 x SSC).

PMT1076 was restriction mapped and the DNA sequence determined by the Maxam-Gilbert method. The sequence covering the open reading frame is shown in SEQ ID No. 1. The open reading frame is seen to encode a putative signal sequence of 21 amino acids (according to the von Heine rules (von Heijne, G. (1986)) and furthermore a propeptide of 10 amino acids preceding the N-terminal of the mature lipase. The last two amino acids of the 15 propeptide are Arg Arg, i.e. a typical cleavage site for endoproteolytic processing by enzymes of the S. cerevisiae KEX-2 type. The amino acid composition of the mature protein (starting at position 32) encoded by the DNA sequence is in agreement with the amino acid composition determined for C. antarctica lipase A, cf. the following table:

20

Table I

Amino acid composition of *C. antarctica* lipase A (CALIP)

	Deduced from DNA sequence	By amino acid analysis (MC)
5 Ala	50	47
Arg	9	9
Asp/AsN	35	36
Cys	4	4
Gln/GlN	35	36
10 Gly	28	31
His	6	6
Ile	26	24
Leu	29	30
Lys	17	17
15 Met	2	3
Phe	20	19
Pro	33	33
Ser	26	27
Thr	27	28
20 Trp	5	4
Tyr	18	16
Val	27	26

Through a number of standard plasmid manipulations (Maniatis et al., 1982) illustrated in Figs. 2 and 3, the open reading frame 25 of *C. antarctica* lipase A was placed in the correct orientation between the alpha-amylase promoter of *A. oryzae* and the glucoamylase transcription terminator of *A. niger*. The resulting expression plasmid pMT1229 was transformed into *A. oryzae* A1560 as described in EP 305,216. Transformants were isolated 30 and grown as described in the above cited patents and the culture supernatants were analyzed for the presence of *C. antarctica* lipase A.

EXAMPLE 2

Construction of a plasmid expressing the F135W variant of Candida antarctica lipase A

A 246 bp BamHI/BssHII fragment was synthesized in vitro on the basis of the nucleotide sequence of pMT1229 using oligonucleotide primers 3116 and 3117 in a PCR reaction. The primer 3117 includes a BssHII restriction site and a mutation in the 135 phe codon (TTC) to trp codon (TGG) which is marked with stars.

Oligonucleotide primer 3116 (F135W:256-276) (SEQ ID No. 8)
10 5'-CAG AAC GAG GCG GTG GCC GAC-3'

Oligonucleotide primer 3117 (F135W:566-487) (SEQ ID No. 9)
5'-TTC TTG AGC GCG CGG ATG CCG TCG AGG ATA GCC ATG CCC TCT TCG
TAG CCA GCG ATG AAG GCG GCT TTC* C*AG CCT TCG TG-3'

The PCR reaction was performed by mixing the following components and incubating the mixture in a HYBAID™ thermal reactor.

Template pMT1229	10 ng/μl	1 μl
H ₂ O		46.5 μl
10 x PCR buffer		10 μl
20 2 mM dATP		10 μl
2 mM dTTP		10 μl
2 mM dCTP		10 μl
2 mM dGTP		10 μl
primer 3116	50.5 pmol/μl	1 μl
25 primer 3117	70.5 pmol/μl	1 μl
Taq polymerase		0.5 μl
Parafin oil		50 μl
Step I	94°C	2 min.
Step II	94°C	30 sec.
30	50°C	30 sec.
	72°C	2 min.
Step III	72°C	5 min.
		1 cycle
		30 cycle
		1 cycle

The resulting 310 bp fragment was isolated from a 2% agarose gel after electrophoresis and digested with BamHI and BssHII restriction enzymes. The resulting 264 bp BamHI/BssHII frag-

ment was likewise isolated from 2% agarose gel. This fragment was then ligated with

	pMT1229	BamHI/XbaI	0.3 kb
	pMT1229	BssHII/SphI	0.5 kb
5	pMT1229	SphI/XbaI	5.0 kb

The ligated DNA was transformed into *E. coli* strain MT172. Transformants which contained correct inserts were selected and their DNA sequence was determined by use of Sequenase (United States Biochemical Corporation). One resulting plasmid (pME-
10 1178) contained a mutation in the amino acid position 135 (phe was mutated to trp).

pME1178 was cotransformed with pToC90 which included the *amdS* gene from *A. nidulans* as a selective marker into the *A. oryzae* A1560 strain using the procedure described in WO 91/17243. *A.*
15 *oryzae* transformants were reisolated twice on selective plates and stable transformants were characterized by rocket immunoelectrophoresis, using anti-*Candida* lipase A antibody. *Candida* lipase A produced by a transformant (strain MEA65) was further analyzed for specific activity.

20 EXAMPLE 3

Construction of a plasmid expressing the F139W variant of *Candida antarctica* lipase A

A 246 bp BamHI/BssHII fragment was synthesized in vitro on the basis of the nucleotide sequence of the plasmid pMT1229 using
25 oligonucleotide primers 3116 and 3826 in a PCR reaction. The primer 3826 includes a BssHII restriction site and a mutation in the 139 phe codon (TTC) to trp codon (TGG) which is marked with stars.

Oligonucleotide primer 3116 is shown in Example 2.

Oligonucleotide primer 3826 (F139W:566-487) (SEQ ID No. 10)
5'-TTC TTG AGC GCG CGG ATG CCG TCG AGG ATA GCC ATG CCC TCT TCG
TAG CCA GCG ATC* C*AG GCG GCT TTG AAG CCT TCG TG-3'

5 A PCR reaction was performed by the method described in Example
2. The 310 bp fragment was isolated from 2% agarose gel after
electrophoresis and digested by BamHI and BssHII restriction
enzymes. The resulting 264 bp BamHI/BssHII fragment was
likewise isolated from 2% agarose gel. This fragment was then
10 ligated with

pMT1229	BamHI/XbaI	0.3 kb
pMT1229	BssHII/SphI	0.5 kb
pMT1229	SphI/XbaI	5.0 kb

The ligated DNA was transformed into E. coli strain MT172.
15 Transformants which contained correct inserts were selected and
their DNA sequence was determined by use of Sequenase (United
States Biochemical Corporation). One resulting plasmid (pME-
1229) contained a mutation in the amino acid position 139 (phe
was mutated to trp).

20 pME1229 was cotransformed with pToC90 which included the amdS
gene from *A. nidulans* as a selective marker into *A. oryzae* A
1560 strain. *A. oryzae* transformants were reisolated twice on
selective plates and enzyme activity of a stable transformant
(MEA155) was analyzed by using tributylene as a substrate as
25 described in Example 5.

,EXAMPLE 4

Construction of a plasmid expressing the F135W/F139W variant of Candida antarctica lipase A

A 246 bp BamHI/BssHII fragment was synthesized in vitro using 5 oligonucleotide primers 3116 and 4224 by a PCR reaction. The primer 4224 includes a BssHII restriction site and mutations in the 135 and 139 codons (TTC) to trp codons (TGG) which are marked with stars.

The oligonucleotide primer 3116 is shown in Example 2.

10 Oligonucleotide primer 4224 (F135W:566-487) (SEQ ID No. 11)
 5'-TTC TTG AGC GCG CGG ATG CCG TCG AGG ATA GCC ATG CCC TCT TCG
 TAG CCA GCG ATC* C*AG GCG GCT TTC* C*AG CCT TCG TG-3'

PCR reaction was performed by using the method shown in Example 2. The 310 bp fragment was isolated from a 2% agarose gel after 15 electrophoresis and digested with BamHI and BssHII restriction enzymes. The resulting 264 bp BamHI/BssHII fragment was likewise isolated from a 2% agarose gel. This fragment was then ligated with

20	pMT1229	BamHI/XbaI	0.3 kb
	pMT1229	BssHII/SphI	0.5 kb
	pMT1229	SphI/XbaI	5.0 kb

The ligated DNA was transformed into *E. coli* MT172. Transformants which contained inserts were selected and their DNA sequence was determined by use of Sequenase. One resulting 25 plasmid (pME1230) contained two mutations in the amino acid positions 135 and 139 (phe was mutated to trp).

pME1230 was cotransformed with pToC90 which included the *amdS* gene from *A. nidulans* as a selective marker into *A. oryzae* A 1560 strain. *A. oryzae* transformants were reisolated twice on

, selective plates and enzyme activity of stable transformants were analyzed by using tributylene as a substrate as described in Example 5.

EXAMPLE 5

5 Purification of C. antarctica lipase A variants F139W and F135W/F139W and comparison of specific activity with their parent wild type C. antarctica lipase A

The lipase variants and the parent lipase produced as described in Examples 3, 4 and 1, respectively, were purified using the
10 following 4 step standard purification procedure.

Step 1: The fermentation broth containing the lipase and lipase variant, respectively, obtained by culturing the transformed A. oryzae cells described in Examples 1 and 3 above, was centrifuged, and the supernatant was adjusted to pH 7. Ionic strength
15 was adjusted to 2 mSi. DEAE-Sephadex A-50 (Pharmacia) gel was swollen and equilibrated in 25 mM Tris acetate buffer pH 7. The fermentation supernatant was passed through DEAE-Sephadex A-50 on scintered glass funnel. The effluent containing lipase activity was collected and adjusted to 0.8 M ammonium acetate.

20 Step 2: An appropriate column was packed with TSK gel Butyl-Toyopearl 650 C and equilibrated with 0.8 M ammonium acetate. The effluent containing lipase activity was applied on the column. The bound material was eluted with water.

Step 3: The lipase-containing eluate was then applied on a
25 Highperformance Q-Sepharose column. Lipase activity was collected as effluent. The lipases purified by this method were concentrated to an Optical Density of 1 at 280 nm.

The purity of the lipases was checked by SDS-PAGE showing one band with an molecular weight of about 45 kD. The lipase

activity was determined by use of the method outlined above in the section "General methods".

The lipase activity of the parent wild type lipase was 300 LU/OD₂₈₀ as compared to 1200 LU/OD₂₈₀ for the lipase variant F139W. On the basis of OD₂₈₀ absorption without correction for the inserted tryptophan, the specific activity of the mutant was at least four times higher with the assay used. The lipase activity of the lipase variant F135W/F139W was 1400 LU/OD₂₈₀ (without correction for the two additional tryptophans).

10 EXAMPLE 6

Thermostability of *Candida antarctica* lipase A and the mutant F139W thereof

The thermostability of the *C. antarctica* lipase A and the *C. antarctica* lipase A variant, was examined by Differential Scanning Calorimetry (DSC) at different pH values. Using this technique, the thermal denaturation temperature, T_d , is determined by heating an enzyme solution at a constant programmed rate.

More specifically, the Differential Scanning Calorimeter, MC-20 2D, from MicroCal Inc. was used for the investigations. Enzyme solutions were prepared in 50 mM buffer solutions, cf. the tables below. The enzyme concentration ranged between 0.6 and 0.9 mg/ml, and a total volume of about 1.2 ml was used for each experiment. All samples were heated from 25°C to 90°C at a scan rate of 90°C/hr.

The results obtained from the analysis is shown in the table below:

C. ant. lipase A (WT)

pH	Buffer (50 mM)	Denaturation temperature ¹⁾
4.5	Acetate	96°C
5	Acetate	95°C
7	TRIS	93°C

C. ant. lipase A mutant (F139W)

10 pH	Buffer (50 mM)	Denaturation temperature ¹⁾
5	Acetate	84°C
7	TRIS	82°C

15 ¹⁾ Temperature, at which approximately half the enzyme molecules present have been denatured thermally during heating

20 The above results show that the pH-optimum for the thermostability of *C. antarctica* lipase A and the F139W variant is unusually low and that both enzymes are very thermostable below pH 7. Within the investigated range the thermostability of both the Wild Type and the mutant F139W continues to increase as pH is lowered. This makes both lipases very well suited for hydrolysis/synthesis at unusually high temperatures at relatively low pH values.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

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 (B) STREET: Novo Alle
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 (E) COUNTRY: DENMARK
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 10 (G) TELEPHONE: +45 44448888
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 (I) TELEX: 37304

(ii) TITLE OF INVENTION: Lipase Variants

(iii) NUMBER OF SEQUENCES: 11

15 (iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
 (B) COMPUTER: IBM PC compatible
 (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 20 (EPO) (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- 25 (A) LENGTH: 1389 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

30 (v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Candida antarctica
 (C) INDIVIDUAL ISOLATE: DSM 3855

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```

35 ATGGGAGTGT CCTGGGCTC CATCACTGG CTGCTTGGGG OGGCAAOGGC GGCTGTGCTC      60
   GGGGCTOOGG OGGCOGAGAC GCTGGACOGA OGGGGGGGGC TGCCCAACCC CTAAGAAGAT      120
   CCTTCTACA OGAOGCCATC CAACATGGC ACGTTTGGCA AGGGCCAGGT GATCCAATCT      180
   OGCAAGGTGC CCAOGGACAT OGGCAAOGCC AACAAOGCTG CGTGGTTCCA GCTGCAGTAC      240

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	CGCACCA	ATA	GCAGAA	CGAGG	GGGTG	GCOGA	OGTGG	CCACOG	TGTG	GAT	CCGGCC	300
	AAGCC	CGCT	CGCAA	GATC	TTTTG	TACC	AGTCT	ACG	AGGAT	GC	CACGG	360
	GACT	TGCTC	CGAG	CTAC	CTACT	GGAT	TGGAC	AGOO	GAACA	AA	GGTGA	420
	GTGCT	OGACA	CGCC	ATCAT	CATOG	GGCTG	GCG	CTG	CAGC	AGGG	CTACTA	480
5	TOOG	ACCAOG	AAGG	CTTCAA	AGCOG	CCCTC	ATOG	CTGG	CT	ACGA	AGAGGG	540
	CTOG	AGGCA	TOOG	CGGCT	CAAGA	ACTAC	CAGA	ACCTG	CAT	OGAC	AG	600
	CTG	AGGGCT	ACAG	TGGGG	AGCT	CAAGC	ACOG	TG	GGG	CGACT	TOGCT	660
	TAC	GGCCCG	AGCT	CAACAT	TGTO	GGTGT	TOG	CAOG	GG	GCAOG	CCOGT	720
	GAC	ACCTTTA	CAT	CTCTCA	AA	CGGG	ACCC	TTOG	COGGCT	TTG	CCCTGGC	780
10	GGT	CTCTG	CTG	CTAT	CC	TGAT	ATGG	AGCT	TCATTG	AGG	CCOGATT	840
	GGT	CAGOGGA	CGCT	CAAGCA	GATC	OGOGG	CGT	GGCT	CTCT	GCC	TGCGCA	900
	ACCT	ACCCCT	TOCT	CAAOGT	CTT	CTOG	CTG	GTCA	AGACA	CGA	ACCTGCT	960
	COG	ATOGCTA	GCAT	CTCAA	GCAG	GAGACT	GTEG	TCCAGG	CGA	AGOGAG	CTACA	1020
	TOGG	TGCCA	AGTT	CCOGG	CTT	CAT	CTGG	CAT	GOGAT	CCG	ACGAGAT	1080
15	CAG	CTGOGG	CTAC	CTAOGT	CAAG	GAGCAA	TGTG	CCAA	GG	GOG	CAACAT	1140
	CCCT	ACCCGA	TOG	COGAGCA	CC	TCACOG	CC	GAGAT	CTTTG	GT	CTGGTG	1200
	TTT	ATCAAGC	AAGC	CTTGA	CGG	CACCACA	CCCA	AGGTGA	TC	TGOG	GCAC	1260
	GCT	ATOGCTG	GCAT	CACCAC	GCC	CTOGG	G	ACCA	AGTGC	TGG	GTGGA	1320
	CAG	CTGOGCA	GCT	OGAOGG	CAAG	CAGAGT	GOG	TGOGCA	AGC	CCCTTGG	CCCATCACA	1380
20	CCAC	CTTAG										1389

(2) INFORMATION FOR SEQ ID NO: 2:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 463 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (iii) HYPOTHETICAL: NO
- (iii) ANTI-SENSE: NO
- (v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Candida antarctica*
 (C) INDIVIDUAL ISOLATE: DSM 3855

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

5 Met Arg Val Ser Leu Arg Ser Ile Thr Ser Leu Leu Ala Ala Ala Thr
 1 5 10 15
 Ala Ala Val Leu Ala Ala Pro Ala Ala Glu Thr Leu Asp Arg Arg Ala
 20 25 30
 10 Ala Leu Pro Asn Pro Tyr Asp Asp Pro Phe Tyr Thr Thr Pro Ser Asn
 35 40 45
 Ile Gly Thr Phe Ala Lys Gly Gln Val Ile Gln Ser Arg Lys Val Pro
 50 55 60
 Thr Asp Ile Gly Asn Ala Asn Asn Ala Ala Ser Phe Gln Leu Gln Tyr
 65 70 75 80
 15 Arg Thr Thr Asn Thr Gln Asn Glu Ala Val Ala Asp Val Ala Thr Val
 85 90 95
 Trp Ile Pro Ala Lys Pro Ala Ser Pro Pro Lys Ile Phe Ser Tyr Gln
 100 105 110
 20 Val Tyr Glu Asp Ala Thr Ala Leu Asp Cys Ala Pro Ser Tyr Ser Tyr
 115 120 125
 Leu Thr Gly Leu Asp Gln Pro Asn Lys Val Thr Ala Val Leu Asp Thr
 130 135 140
 Pro Ile Ile Ile Gly Trp Ala Leu Gln Gln Gly Tyr Tyr Val Val Ser
 145 150 155 160
 25 Ser Asp His Glu Gly Phe Lys Ala Ala Phe Ile Ala Gly Tyr Glu Glu
 165 170 175
 Gly Met Ala Ile Leu Asp Gly Ile Arg Ala Leu Lys Asn Tyr Gln Asn
 180 185 190
 30 Leu Pro Ser Asp Ser Lys Val Ala Leu Glu Gly Tyr Ser Gly Gly Ala
 195 200 205
 His Ala Thr Val Trp Ala Thr Ser Leu Ala Glu Ser Tyr Ala Pro Glu
 210 215 220
 Leu Asn Ile Val Gly Ala Ser His Gly Gly Thr Pro Val Ser Ala Lys
 225 230 235 240
 35 Asp Thr Phe Thr Phe Leu Asn Gly Gly Pro Phe Ala Gly Phe Ala Leu
 245 250 255
 Ala Gly Val Ser Gly Leu Ser Leu Ala His Pro Asp Met Glu Ser Phe

		260				265					270					
	Ile	Glu	Ala	Arg	Leu	Asn	Ala	Lys	Gly	Gln	Arg	Thr	Leu	Lys	Gln	Ile
		275						280					285			
5	Arg	Gly	Arg	Gly	Phe	Cys	Leu	Pro	Gln	Val	Val	Leu	Thr	Tyr	Pro	Phe
		290					295					300				
	Leu	Asn	Val	Phe	Ser	Leu	Val	Asn	Asp	Thr	Asn	Leu	Leu	Asn	Glu	Ala
	305					310					315				320	
	Pro	Ile	Ala	Ser	Ile	Leu	Lys	Gln	Glu	Thr	Val	Val	Gln	Ala	Glu	Ala
					325					330					335	
10	Ser	Tyr	Thr	Val	Ser	Val	Pro	Lys	Phe	Pro	Arg	Phe	Ile	Trp	His	Ala
			340						345					350		
	Ile	Pro	Asp	Glu	Ile	Val	Pro	Tyr	Gln	Pro	Ala	Ala	Thr	Tyr	Val	Lys
			355					360						365		
15	Glu	Gln	Cys	Ala	Lys	Gly	Ala	Asn	Ile	Asn	Phe	Ser	Pro	Tyr	Pro	Ile
		370					375					380				
	Ala	Glu	His	Leu	Thr	Ala	Glu	Ile	Phe	Gly	Leu	Val	Pro	Ser	Leu	Trp
	385					390					395				400	
	Phe	Ile	Lys	Gln	Ala	Phe	Asp	Gly	Thr	Thr	Pro	Lys	Val	Ile	Cys	Gly
					405					410					415	
20	Thr	Pro	Ile	Pro	Ala	Ile	Ala	Gly	Ile	Thr	Thr	Pro	Ser	Ala	Asp	Gln
				420					425					430		
	Val	Leu	Gly	Ser	Asp	Leu	Ala	Asn	Gln	Leu	Arg	Ser	Leu	Asp	Gly	Lys
			435					440					445			
25	Gln	Ser	Ala	Phe	Gly	Lys	Pro	Phe	Gly	Pro	Ile	Thr	Pro	Pro	Glx	
	450						455					460				

(2) INFORMATION FOR SEQ ID NO: 3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 277 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (iii) HYPOTHETICAL: NO
- (iii) ANTI-SENSE: NO
- 35 (v) FRAGMENT TYPE: internal
- (vi) ORIGINAL SOURCE:

(A) ORGANISM: *Pseudomonas fragi*

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Met Asp Asp Ser Val Asn Thr Arg Tyr Pro Ile Leu Leu Val His Gly
 1 5 10 15

5 Leu Phe Gly Phe Asp Arg Ile Gly Ser His His Tyr Phe His Gly Ile
 20 25 30

Lys Gln Ala Leu Asn Glu Cys Gly Ala Ser Val Phe Val Pro Ile Ile
 35 40 45

10 Ser Ala Ala Asn Asp Asn Glu Ala Arg Gly Asp Gln Leu Leu Lys Gln
 50 55 60

Ile His Asn Leu Arg Arg Gln Val Gly Ala Gln Arg Val Asn Leu Ile
 65 70 75 80

Gly His Ser Gln Gly Ala Leu Thr Ala Arg Tyr Val Ala Ala Ile Ala
 85 90 95

15 Pro Glu Leu Ile Ala Ser Val Thr Ser Val Ser Gly Pro Asn His Gly
 100 105 110

Ser Glu Leu Ala Asp Arg Leu Arg Leu Ala Phe Val Pro Gly Arg Leu
 115 120 125

20 Gly Glu Thr Val Ala Ala Ala Leu Thr Thr Ser Phe Ser Ala Phe Leu
 130 135 140

Ser Ala Leu Ser Gly His Pro Arg Leu Pro Gln Asn Ala Leu Asn Ala
 145 150 155 160

Leu Asn Ala Leu Thr Thr Asp Gly Val Ala Ala Phe Asn Arg Gln Tyr
 165 170 175

25 Pro Gln Gly Leu Pro Asp Arg Trp Gly Gly Met Gly Pro Ala Gln Val
 180 185 190

Asn Ala Val His Tyr Tyr Ser Trp Ser Gly Ile Ile Lys Gly Ser Arg
 195 200 205

30 Leu Ala Glu Ser Leu Asn Leu Leu Asp Pro Leu His Asn Ala Leu Arg
 210 215 220

Val Phe Asp Ser Phe Phe Thr Arg Glu Thr Arg Glu Asn Asp Gly Met
 225 230 235 240

Val Gly Arg Phe Ser Ser His Leu Gly Gln Val Ile Arg Ser Asp Tyr
 245 250 255

35 Pro Leu Asp His Leu Asp Thr Ile Asn His Met Ala Arg Gly Ser Ala
 260 265 270

Gly Ala Ser Thr Arg
275

(2) INFORMATION FOR SEQ ID NO: 4:

- (i) SEQUENCE CHARACTERISTICS:
5 (A) LENGTH: 269 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- 10 (iii) HYPOTHETICAL: NO
(iii) ANTI-SENSE: NO
(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:
(A) ORGANISM: *Rhizomucor miehei*
- 15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:
- Ser Ile Asp Gly Gly Ile Arg Ala Ala Thr Ser Gln Glu Ile Asn Glu
1 5 10 15
Leu Thr Tyr Tyr Thr Thr Leu Ser Ala Asn Ser Tyr Cys Arg Thr Val
20 25 30
Ile Pro Gly Ala Thr Trp Asp Cys Ile His Cys Asp Ala Thr Glu Asp
35 40 45
Leu Lys Ile Ile Lys Thr Trp Ser Thr Leu Ile Tyr Asp Thr Asn Ala
50 55 60
Met Val Ala Arg Gly Asp Ser Glu Lys Thr Ile Tyr Ile Val Phe Arg
25 65 70 75 80
Gly Ser Ser Ser Ile Arg Asn Trp Ile Ala Asp Leu Thr Phe Val Pro
85 90 95
Val Ser Tyr Pro Pro Val Ser Gly Thr Lys Val His Lys Gly Phe Leu
100 105 110
30 Asp Ser Tyr Gly Glu Val Gln Asn Glu Leu Val Ala Thr Val Leu Asp
115 120 125
Gln Phe Lys Gln Tyr Pro Ser Tyr Lys Val Ala Val Thr Gly His Ser
130 135 140
35 Leu Gly Gly Ala Thr Ala Leu Leu Cys Ala Leu Gly Leu Tyr Gln Arg
145 150 155 160
Glu Glu Gly Leu Ser Ser Ser Asn Leu Phe Leu Tyr Thr Gln Gly Gln

165 170 175
 Pro Arg Val Gly Asp Pro Ala Phe Ala Asn Tyr Val Val Ser Thr Gly
 180 185 190
 5 Ile Pro Tyr Arg Arg Thr Val Asn Glu Arg Asp Ile Val Pro His Leu
 195 200 205
 Pro Pro Ala Ala Phe Gly Phe Leu His Ala Gly Glu Glu Tyr Trp Ile
 210 215 220
 Thr Asp Asn Ser Pro Glu Thr Val Gln Val Cys Thr Ser Asp Leu Glu
 225 230 235 240
 10 Thr Ser Asp Cys Ser Asn Ser Ile Val Pro Phe Thr Ser Val Leu Asp
 245 250 255
 His Leu Ser Tyr Phe Gly Ile Asn Thr Gly Leu Cys Ser
 260 265

(2) INFORMATION FOR SEQ ID NO: 5:

- 15 (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 297 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- 20 (ii) MOLECULE TYPE: protein
- (iii) HYPOTHETICAL: NO
- (iii) ANTI-SENSE: NO
- (v) FRAGMENT TYPE: internal
- (vi) ORIGINAL SOURCE:
 - 25 (A) ORGANISM: Rhizopus niveus
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Asp Asp Asn Leu Val Gly Gly Met Thr Leu Asp Leu Pro Ser Asp Ala
 1 5 10 15
 30 Pro Pro Ile Ser Leu Ser Ser Ser Thr Asn Ser Ala Ser Asp Gly Gly
 20 25 30
 Lys Val Val Ala Ala Thr Thr Ala Gln Ile Gln Glu Phe Thr Lys Tyr
 35 40 45
 Ala Gly Ile Ala Ala Thr Ala Tyr Cys Arg Ser Val Val Pro Gly Asn
 50 55 60
 35 Lys Trp Asp Cys Val Gln Cys Gln Lys Trp Val Pro Asp Gly Lys Ile
 65 70 75 80

Ile Thr Thr Phe Thr Ser Leu Leu Ser Asp Thr Asn Gly Tyr Val Leu
85 90 95

Arg Ser Asp Lys Gln Lys Thr Ile Tyr Leu Val Phe Arg Gly Thr Asn
100 105 110

5 Ser Phe Arg Ser Ala Ile Thr Asp Ile Val Phe Asn Phe Ser Asp Tyr
115 120 125

Lys Pro Val Lys Gly Ala Lys Val His Ala Gly Phe Leu Ser Ser Tyr
130 135 140

10 Glu Gln Val Val Asn Asp Tyr Phe Pro Val Val Gln Glu Gln Leu Thr
145 150 155 160

Ala His Pro Thr Tyr Lys Val Ile Val Thr Gly His Ser Leu Gly Gly
165 170 175

Ala Gln Ala Leu Leu Ala Gly Met Asp Leu Tyr Gln Arg Glu Pro Arg
180 185 190

15 Leu Ser Pro Lys Asn Leu Ser Ile Phe Thr Val Gly Gly Pro Arg Val
195 200 205

Gly Asn Pro Thr Phe Ala Tyr Tyr Val Glu Ser Thr Gly Ile Pro Phe
210 215 220

20 Gln Arg Thr Val His Lys Arg Asp Ile Val Pro His Val Pro Pro Gln
225 230 235 240

Ser Phe Gly Phe Leu His Pro Gly Val Glu Ser Trp Ile Lys Ser Gly
245 250 255

Thr Ser Asn Val Gln Ile Cys Thr Ser Glu Ile Glu Thr Lys Asp Cys
260 265 270

25 Ser Asn Ser Ile Val Pro Phe Thr Ser Ile Leu Asp His Leu Ser Tyr
275 280 285

Phe Asp Ile Asn Glu Gly Ser Cys Leu
290 295

(2) INFORMATION FOR SEQ ID NO: 6:

- 30 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 44 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- 35 (ii) MOLECULE TYPE: DNA (synthetic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

5 GCTGCTCTGCG CTAACCCCTA CGAYGAYCCT TTCTACACCA CCCC

44

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 17 base pairs
 (B) TYPE: nucleic acid
 10 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

15 (v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

AAYCCNTAYG AYGAYCC

17

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
 20 (A) LENGTH: 21 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

25 (iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

CAGAACGAGG OGGTGGCOGA C

21

30 (2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 80 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: DNA (synthetic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

10 TTCITGAGCG CGGGATGCC GTGAGGATA GCCATGCOCT CTTOGTAGCC AGCGATGAAG 60
 GGGCITTC AGCCTTOGTG 80

(2) INFORMATION FOR SEQ ID NO: 10:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 80 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

15

(ii) MOLECULE TYPE: DNA (synthetic)

(iii) HYPOTHETICAL: NO

20 (iii) ANTI-SENSE: YES

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

TTCITGAGCG CGGGATGCC GTGAGGATA GCCATGCOCT CTTOGTAGCC AGCGATCCAG 60
 GGGCITTIGA AGCCTTOGTG 80

25 (2) INFORMATION FOR SEQ ID NO: 11:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 80 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

30

(ii) MOLECULE TYPE: DNA (synthetic)

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: YES

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

TTCTTGAGCG CGGGATGCC GTGAGGATA GCCATGCCCT CTTGTAGCC AGGATCCAG 60
5 GGGCTTTC AGCCITGTG 80

CLAIMS

1. A lipase variant of a parent lipase comprising a trypsin-like catalytic triad including an active serine located in a predominantly hydrophobic, elongated binding pocket of the lipase molecule and, located in a critical position of a lipid contact zone of the lipase structure, an amino acid residue different from an aromatic amino acid residue, which amino acid residue interacts with a lipid substrate at or during hydrolysis, in which lipase variant said amino acid residue has been replaced by an aromatic amino acid residue so as to confer to the variant an increased specific activity as compared to that of the parent lipase.
2. A lipase variant according to claim 1, wherein the aromatic amino acid residue to be inserted in the critical position is selected from the group consisting of tryptophan, phenylalanine, tyrosine and histidine.
3. A lipase variant according to claim 1 or 2, in which said amino acid residue different from an aromatic amino acid residue is a phenylalanine residue.
4. A lipase variant according to any of claims 1-3, in which the amino acid residue located in the critical position of the lipase is different from tryptophan, and said amino acid residue has been replaced with a tryptophan residue.
5. A lipase variant according to any of the preceding claims, wherein the parent lipase is selected from a microbial or a mammalian lipase.
6. A lipase variant according to claim 5, wherein the parent lipase is a yeast lipase.
7. A lipase variant according to claim 6, wherein the parent lipase is derived from a strain of Candida antarctica.

8. A lipase variant according to claim 7, wherein the parent lipase is lipase A of C. antarctica.
9. A lipase variant according to claim 8, which has the amino acid sequence shown in SEQ ID No. 1, in which the phenylalanine 139 of the parent lipase has been replaced by a tryptophan residue, or in which the phenylalanine 135 and 139 of the parent lipase have been replaced by tryptophan residues.
10. A lipase variant according to claim 5, in which the parent lipase is a bacterial lipase.
- 10 11. A lipase variant according to claim 10, wherein the parent lipase is derived from a strain of Pseudomonas.
12. A lipase variant according to claim 11, which is derived from a strain of Ps. fragi.
13. A lipase variant according to claim 12, which has the amino acid sequence shown in SEQ ID No. 3 in which the phenylalanine 29 of the parent lipase has been replaced by a tryptophan residue.
14. A lipase variant according to claim 5, wherein the parent lipase is selected from a fungal lipase, a human lipase, a murine lipase, a rat lipase or a canine lipase.
15. A C. antarctica lipase A essentially free from other substances from C. antarctica, which comprises the amino acid sequence shown in SEQ ID No. 2, or a variant of said lipase which
- 25 1) has lipase activity,
- 2) reacts with an antibody reactive with at least one epitope of C. antarctica lipase A having the amino acid sequence SEQ ID No. 2, and/or

- 3) is encoded by a nucleotide sequence which hybridizes with an oligonucleotide probe prepared on the basis of the full or partial nucleotide sequence shown in SEQ ID No. 1 encoding the C. antarctica lipase A.
- 5 16. A DNA sequence encoding C. antarctica lipase A having the amino acid sequence shown in SEQ ID No. 2 or a modification of said DNA sequence which encodes a variant of C. antarctica lipase A which
- 1) has lipase activity,
 - 10 2) reacts with an antibody reactive with at least one epitope of the C. antarctica lipase A having the amino acid sequence SEQ ID No. 2, and/or
 - 3) is encoded by a nucleotide sequence which hybridizes with an oligonucleotide probe prepared on the basis of the full or
15 partial nucleotide sequence shown in SEQ ID No. 1 encoding the C. antarctica lipase A.
17. A DNA construct comprising a DNA sequence encoding a lipase variant according to any of claims 1-14 or C. antarctica lipase A according to claim 15.
- 20 18. A recombinant expression vector which carries a DNA construct according to claim 17.
19. A cell which is transformed with a DNA construct according to claim 17 or a vector according to claim 18.
20. A cell according to claim 19 which is a fungal cell, e.g.
25 belonging to the genus Aspergillus, such as A. niger, A. oryzae, or A. nidulans; a yeast cell, e.g. belonging to a strain of Saccharomyces, such as S. cerevisiae, or a methylotrophic yeast from the genera Hansenula, such as H. polymorpha, or Phichia, such as P. pastoris; or a bacterial cell,

, e.g. belonging to a strain of Bacillus, such as B. subtilis, B. licheniformis or B. lentus, or to a strain of Escherichia, such as E. coli.

21. A method of producing a lipase variant according to any of
5 claims 1-14, wherein a cell according to claim 19 or 20 is
cultured under conditions conducive to the production of the
lipase variant, and the lipase variant is subsequently rec-
overed from the culture.

22. Use of a lipase variant according to any of claims 1-14 or
10 the C. antarctica lipase A or a variant thereof according to
claim 15 in ester hydrolysis, ester synthesis or interesterifi-
cation.

23. Use of a lipase variant according to any of claims 1-14 or
the C. antarctica lipase A or a variant thereof according to
15 claim 15 for avoiding pitch trouble in a process for the
production of mechanical pulp or a paper-making process using
mechanical pulp.